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Cover letter

*Response to Reviewer Comments

Anion retention in hydrophilic interaction liquid chromatography with TFA studied.

Strong retention of cationic solutes with hydride columns in TFA is moderated.

Unusual retention effects probably not caused by metal cation adsorption.

Methane sulfonic acid gives cationic retention i.e. different selectivity to TFA.

Ionic strength of mobile phase an important influential factor in these effects.

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6 Effect of mobile phase additives on solute retention at low aqueous pH in hydrophilic
7 interaction liquid chromatography.
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24 **Abstract**

26 Trifluoracetic acid (TFA) added to the aqueous acetonitrile mobile phase induces some
27 unexpected changes in the ionic component of retention in hydrophilic interaction
28 separations when using Type B silica and amide-bonded silica columns. TFA use results in
29 anion exchange properties which contrast with the cation exchange typically found with
30 ammonium salt buffers. The significant cation exchange properties of silica hydride columns
31 are also moderated by TFA. Similar behaviour was shown in a metal- free amide column
32 operated on a system washed with a metal complexing agent, suggesting that adsorbed
33 metal cations were not responsible for this anion exchange behaviour. It is possible that the
34 column surface acquires some positive charges at the low pH of TFA. A surprising reversal
35 of the properties of the columns back to predominately cation exchange behaviour was
36 shown using methanesulfonic acid (MSA), which appears to be a stronger acid than TFA in
37 high concentrations of acetonitrile. MSA maintains sufficient ionic strength in the mobile
38 phase even at low concentrations, giving good peak shape, which could be useful for mass
39 spectrometry detection. Besides giving different selectivity to TFA, MSA also gives different
40 selectivity to that of ammonium salt buffers, suggesting it may be useful in manipulating the
41 selectivity of a separation. Similar changes to the selectivity with TFA could be achieved by
42 adding neutral methylsulfonate salts to the TFA mobile phase. While it is possible that
43 methylsulfonate ions are retained on the stationary phase surface, experiments using ion
44 pair reagents of opposite charge yielded the same results as MSA salts. It therefore seems
45 more likely that the higher ionic strength of these solutions negates the influence of charges
46 that may be formed in TFA solutions.

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1. Introduction

Hydrophilic interaction liquid chromatography (HILIC) is becoming increasingly established as an alternative to reversed phase (RP) and ion pair methods for the separation of polar and ionised compounds that may be difficult to retain by these classical procedures. Its applications are widespread, particularly in the biomedical and clinical applications field, and in metabolomics, where many compounds of interest are hydrophilic [1, 2]. Its mechanism is increasingly understood [3-10] and involves partition of solutes between a water layer held on the surface of a polar stationary phase, and the bulk mobile phase, together with adsorption and ionic retention. Using common HILIC mobile phases such as ammonium formate (AF) buffers at acidic $_w^w$ pH, basic solutes typically have increased retention compared with acids, particularly on bare silica HILIC columns. This result can be attributed in part to the higher pH of the AF buffers when measured in the aqueous-organic phase ($_w^s$ pH) and the possibility of interaction of the positively charged basic solute with negatively charged silanols on the stationary phase [6]. Recently, we noted unusual retention effects in HILIC when incorporating stronger acids such as trifluoracetic acid (TFA) or heptafluorobutyric acid (HFBA) into acetonitrile-water mobile phases [11]. These acids produced very different selectivity for ionised acidic and basic solutes compared with AF buffers at similar (aqueous) $_w^w$ pH. For example in TFA, the retention of fully ionised acidic solutes was considerably enhanced relative to that of ionised bases of similar hydrophilicity, thus demonstrating a complete reversal of their order of elution. These unusual retention effects are difficult to explain in detail. They could be due merely to the suppression of underlying silanol ionisation at the low pH of TFA, leading to the predominance of hydrophilic retention of acids, in the absence of repulsion effects. However, it is feasible that at the low $_w^s$ pH of TFA, the silica surface becomes positively charged leading to anion exchange properties that are competitive with the cation exchange properties of silica attributed to silanol dissociation [11]. It is possible that at low pH, hydronium (H_3O^+) ions become incorporated into the tightly bonded immobilised layer of water close to the column surface, or cause further protonation of the stationary phase (e.g. residual silanols) yielding a positive charge. The latter seems a possibility as the point of zero charge (pzc) of silica is considered to be in the region of 2-3, which is in the range of values achieved using 0.1 % TFA [12-14]. Leaching of metal ions (such as Fe^{3+}) from metallic components of the system at the low pH of 0.1 % TFA, could alternatively provide cationic sites responsible for the high retention of ionised acidic solutes.

In this study we have explored further these possibilities for altering retention selectivity, and attempted to throw more light on the processes involved. We investigated the effect of metal ions through the use of a column with no metal components, and the effect of washing with complexing agents. We also studied the behaviour of a silica hydride column (Type C silica) which is claimed to have few silanol groups, to see if it behaved in the same way. According to some authors, hydride columns function by a distinct mechanism from HILIC ("aqueous normal phase", ANP [15]). Hydride columns are claimed to possess a rather thin water layer in HILIC mobile phases, thus giving preponderance of an adsorption over a partition mechanism [15], which has been confirmed by experimental measurement [16]. They have considerable cation exchange properties in the HILIC mode that have been attributed to the adsorption of hydroxyl ions or to a decrease in the amount of adsorbed protons [16]. As the concentration of hydroxyl ions in low pH TFA mobile phases is expected to be small, different retention effects might be expected. We have also studied the use of methanesulfonic acid (MSA) as an alternative to TFA that is also compatible with mass spectrometric detection (MS), and examined the use of various salts in order to elucidate the reasons for any changes in selectivity. Few previous studies have investigated the effects of such additives in HILIC [18, 19].

2.   Experimental.

Experiments were performed with a 1290 binary high pressure mixing instrument with photodiode array detector (0.6 μL flow cell) (Agilent, Waldbronn, Germany) using 5 μL injections. The columns (15 cm x 0.46 cm ID) were XBridge BEH Amide (3.5 μm particle size, pore size 140 Å, surface area 190 m^2/g); XBridge HILIC, (3.5 μm particle size, pore size 136 Å, surface area 183 m^2/g) from Waters, Milford USA, and Cogent Silica C (25 cm x 0.46 cm, 4 μm particle size, pore size 100 Å, surface area 350 m^2/g) from Microsolv (Eatontown, USA). Flow rate was 1.0 mL/min in all experiments. Temperature was maintained at 30 ^{o} C using the Agilent column compartment. Acetonitrile (far UV grade), ammonium formate (AF), ammonium acetate (AA), trifluoroacetic (TFA), methanesulfonic acid (MSA), sodium methanesulfonate (NaMSA) and formic acid (FA) were obtained from Fisher (Loughborough U.K.). Sodium hexane sulfonate and trimethylammonium chloride were obtained from Sigma-Aldrich (Poole, UK). AF buffer was prepared by adjusting an aqueous solution of the salt of appropriate concentration to pH 3.0 with FA. The buffer/additive concentrations referred to are invariably the overall concentrations in the final aqueous-organic mobile phase mixture. The test probes comprised the neutrals uracil,

124   thiourea, uridine, 2-deoxyuridine; the bases cytosine, pyridine, nortriptyline,

125   diphenhydramine, procainamide; the quaternary salt tetramethylphenyl ammonium chloride;

126   the acids 4-OH benzoic, benzenesulfonic, naphthalene-2-sulfonic , p-xylene-2-sulfonic,

127   trihydroxybenzoic acid were all obtained from Sigma-Aldrich; standards were prepared

128   typically at a concentration of 20 mg/L and made up in the exact mobile phase. The pH

129   values of the mobile phase quoted are those either in the aqueous portion of the buffer ($_w^w$

130   pH), as measured in the organic-aqueous combination with the electrode calibrated in

131   aqueous buffers ($_w^s$ pH) or as the true thermodynamic pH, equivalent to that measured in the

132   organic-aqueous solution with the electrode calibrated in organic-aqueous buffers ($_s^s$ pH).

133   pH was measured using a Metrohm 827 meter equipped with Unitrode electrode. Log D

134   values were calculated as the average from 3 different programs: ACD version 12.0 (ACD

135   labs, Toronto, Canada), Marvin (ChemAxon, Budapest, Hungary) and MedChem Designer

136   (Simulations Plus, Lancaster, California, USA). This was done due to the differences given

137   by these programs for the log D values for the same compounds (see supplementary Table

138   S1). Column efficiency was measured at half of peak height. The United States

139   Pharmacopeia (USP) tailing factors were measured at 5% of peak height by dividing the

140   width of the peak by twice the width of its leading edge. The void volume of the columns was

141   determined using toluene as the unretained solute.

142

143   **3.   Results and discussion**

144   *3.1 Unusual retention effects in TFA containing mobile phases.*

145   Fig. 1 shows the separation of a representative mixture of 8 of the 15 test

146   compounds on a BEH hybrid silica column using 5 mM AF in 95% ACN. This mixture

147   contained two neutrals (thiourea, uracil, green numbering), three acidic (4-

148   hydroxybenzoic, 2-NSA, p-XSA, red numbering) and three basic compounds

149   (cytosine, nortriptyline and procainamide, blue numbering). Peak shapes for the

150   entire set of 15 compounds were excellent in this mobile phase giving 18000-25000

151   theoretical plates per column with USP tailing factor <1.3. The high efficiencies can

152   be attributed in part to the appreciable ionic strength of the mobile phase which

153   remains at least 5 mM in high concentrations of acetonitrile due to the presence of

154   the salt. The neutral compounds thiourea (peak 3) and uracil (peak 4) showed rather

155   low retention typical of unbonded silica phases [19]. High retention of the bases

156   nortriptyline (peak 5), cytosine (peak 8) and procainamide (peak 6) together with low

157   retention of the strong acids p-XSA (peak 1) and 2-NSA (peak 2) can be attributed to

158   ionic attraction and repulsion forces with ionised silanol groups on the column.

159   Silanol ionisation may be encouraged by the relatively high pH of the mobile phase

160   (measured as $_w^s$pH = 5.9) compared with the pH of the aqueous portion alone ($_w^w$pH

161   3.0). The silica column using AF buffer can be designated as giving "normal retention

162   behaviour". Nevertheless, cationic retention properties are more moderate on this

163   hybrid phase than on some classical silica phases, which have greater

164   concentrations of ionised silanols [21]. The selectivity of the BEH amide column in

165   the same mobile phase was rather similar, except for slightly greater retention of the

166   strong acids, likely attributable to fewer accessible free silanols on this bonded

167   phase, and reversal of the elution order of cytosine and procainamide (results not

168   shown).

169   Fig. 2a shows the mixture analysed on the same BEH silica column using 0.1

170   %TFA in 95% ACN. Peak shapes were similarly good (18000-25000 plates per

171   column) to those found in AF with little tailing(USP tailing factor <1.3), which can be

172   partially attributed to the reasonable ionic strength of this acid in high concentrations

173   of ACN; peak shapes are much poorer in formic acid solutions which have much

174   lower ionic strength (see below) [11]. However, the selectivity of the separation was

175   completely different to that shown in AF; the longest retention times were shown for

176   p-XSA and 2-NSA, whereas the bases have only small retention. This pattern can be

177   designated as "atypical retention behaviour". Fig. 2b shows the same separation on

178   the BEH amide column, which clearly also demonstrates atypical retention behaviour

179   in even more pronounced fashion. The stronger acids 2-NSA (peak 2) and p-XSA

180   (peak 1), which are negatively charged under the mobile phase conditions, have

181   much longer retention times than are suggested by their moderately negative log

182   D_{pH2} values (-0.48 and -0.73 respectively). In comparison, the base procainamide

183   (peak 6), which has a much more negative log D_{pH2} of -2.68, has k = 0.1 and the less

184   hydrophilic base nortriptyline (log D_{pH2} = 0.94) has k = -0.1, and is thus excluded

185   (lower retention than the void volume marker toluene). Caution is necessary in use of

186   log D values calculated in aqueous solution with behaviour of the compounds in

187   aqueous-organic mobile phases, as both mobile phase pH and solute pK_a will

188   change. The low retention/exclusion of bases cannot be interpreted merely on the

189   suppression of the negative ionisation of silanol groups at the low pH of TFA giving a

190   retention mechanism dominated by hydrophilic retention. Instead the results may be

191   explained by the existence of positive charges on the stationary phase using TFA.

192   As the results for bare silica and bonded phase are similar, it does not seem that the

6

positive charges result from protonation of amide ligands, which would be unlikely anyway considering the low pK_a of such groups. Efficiencies continued to be high on the amide column for most compounds (10000-20000 plates per column) although p-XSA and 2-NSA showed somewhat reduced efficiency (6000-7000 plates) accompanied by some tailing (USP tailing factor ~1.4), as was observed previously [11]. Tailing can be indicative of a mixed retention mechanism where strong interactions are involved. Retention was generally enhanced on the amide column as can be seen for the neutrals thiourea and uracil, attributable to a thicker water layer on such columns [22]. Note that the unusual retention shown in TFA on the silica and amide columns cannot be attributed to variations in the void volume of the column measured with toluene. Indeed the ranges of values for the 15cm silica and amide columns in *all* mobile phases containing 95 % ACN, (including those described in subsequent sections below) were narrow, being 1.76-1.80 mL for the silica and 1.65-1.67 mL for the amide column respectively.

Silica hydride columns have pronounced cation exchange properties with high retention of ionised bases and low retention of ionised acids in AF buffer $_w^w$pH 3 [22]. As the hydride phase possesses a reduced layer of water compared with conventional silica-based HILIC phases, its hydrophilic retention properties may be influenced to a greater extent by adsorption of solutes on the stationary phase through direct hydrogen bonding with surface polar groups rather than partition into a water layer [17]. Thus, it might possibly behave differently in mobile phases containing TFA to the BEH phases. The hydride column was 25cm long rather than 15cm for the amide and bare silica phases. It also had different surface area, pore and particle size, while amide and silica phases were based on the same base material and thus had more comparable similar physical properties to each other (see Experimental section). Nevertheless, Fig. 2c allows a simple visual comparison of the selectivity of the hydride phase with that of the other columns in 95% ACN with 0.1% TFA. While the retention of stronger acid probes was increased and the retention of strong bases decreased compared with AF, there is still quite strong retention of bases like cytosine and procainamide (peaks 8 and 6 respectively) and even for pyridine ($k = 3.8$, not shown in Fig. 2c). Pyridine is moderately hydrophilic (average log D_{pH2} = -1.5) so it is likely that it is retained at least partially by ionic processes. It seems that cation exchange at this low pH is suppressed but not eliminated on the hydride column. Cation exchange could be due to the continued

227   ionisation of acidic silanols that perhaps are formed by hydrolysis through exposure

228   to this acidic mobile phase. The alternative explanation of cationic retention due to

229   the reduced but persistent adsorption of hydroxyl ions (see Introduction) is also

230   possible [18], but would need further examination and explanation, considering the

231   likely very low concentration of hydroxyls at this low mobile phase pH. Peak shapes

232   on this column were reasonable for all 15 solutes, giving efficiencies of 10000-15000

233   plates per (25cm) column and USP tailing factor < 1.3. With this mobile phase, a

234   balance of the retention of cationic and anionic solutes was achieved (Fig. 2c).

235   As 0.1% TFA is soluble even in 100 % ACN, Table 1 shows the possibility of

236   further increasing the retention of acidic compounds, for example on the BEH silica

237   phase. Thus, the retention factor of p-XSA increased from 2.5 to 13.9 to >50 on

238   changing the ACN concentration from 95 to 97 to 98 % ACN.

239

240   *3.2 Influence of metals on atypical retention behaviour.*

241   Poor peak shape for some solutes in HILIC has been shown to be due to detrimental

242   interactions with metals in the system. Examples include nucleotides and

243   hydroxybenzoic acids with vicinal hydroxyl groups when using conventional silica

244   based phases [22], and also for other anionic solutes on a hydride column [23]. In an

245   attempt to discover the possible role of metals for the present study, we obtained a

246   custom-made BEH amide column with a PEEK body and PEEK frits and repeated

247   the analysis of the test mixture using 0.1 % TFA. It is likely that stainless steel

248   column frits are a major source of metal contamination due to their high surface

249   area. The column was tested before and after washing the complete system

250   overnight with a 5 mM solution of EDTA in 50 % ACN (note EDTA at this

251   concentration is not soluble in 95% ACN) in order to complex and remove metal

252   ions. The retention of peaks remained almost identical to Fig. 2b; atypical retention

253   behaviour was again noted with pronounced retention of the acids (peaks 1 and 2)

254   together with low retention or even exclusion of the bases. Caution is necessary in

255   this study as EDTA acts as a complexing agent only in its dissociated form. It is

256   unclear exactly how effective EDTA would be at complexing metals in 50% ACN.

257   Nevertheless, EDTA gave drastic improvement in peak shape in the separation of

258   nucleotides on a conventional BEH amide column in a mobile phase of 70% ACN

259   containing 5 mM AF buffer $_w^w$ pH 3, so there is evidence of its efficacy in HILIC

260   mobile phases [22]. An alternative experiment would be to use the PEEK column,

261 adding a low level of metals to the eluent to see if they had any influence [24].

262 However, our experiments indicate that metal ions are rather unlikely to be the

263 cause of atypical retention behaviour, especially considering the results with other

264 strong acids (see below).

265

266

267 *3.3 Selectivity differences using methanesulfonic acid .*

268 Kadar and co-workers [5] compared the use of TFA and methanesulfonic acid (MSA)

269 additives in ACN-water for the separation of peptides using HILIC. They reported

270 improvements in retention and efficiency for MSA over TFA, but reductions in

271 selectivity, which they attributed to masking of the influence of the amino acid

272 residues of the peptides on their interaction with the stationary phase. However, the

273 complex structures of the peptides and their structural commonality prevented a

274 more in-depth investigation of the effects of MSA on selectivity. As MSA is also

275 compatible with MS detection, we decided to investigate further its possible use in

276 HILIC.

277   We first measured the $_w^s$ pH (the pH in the aqueous-organic mixture with

278 calibration of the electrode in aqueous buffers) of a 13.1 mM solution of MSA

279 (equivalent to the concentration of 0.1 % TFA v/v) as a function of the ACN content

280 over the range 0-95% ACN. Note the concentrations of acids referred to are

281 invariably those in the final aqueous or aqueous-organic mixture. The true

282 thermodynamic $_s^s$pH (equivalent to that measured with the electrode calibrated in

283 aqueous-organic buffers) can be derived using the expression [25]:

284

285 $_s^s\text{pH} = {_w^s}\text{pH} - \delta$ (1)

286

287 where δ is a term that incorporates both the Gibbs free energy for transference of 1

288 mole of protons from the standard state in water to the standard state in the

289 hydroorganic solvent at a given temperature, and the residual liquid junction potential

290 (the difference between the liquid junction potential established during calibration in

291 aqueous solutions, and that in the hydroorganic mixture). Delta was calculated from

292 the empirical equation [25]:

293

294    $\delta = X(a+bT)/(1+cX)$ (2)

296 where T is the temperature on the Celsius scale, X is the ACN concentration and

297 a,b,c are the fitting parameters appropriate to the concentration scale (% v/v in the

298 present case, equation validated over the range 0-90 % ACN, v/v) [25]. Fig. 3a

299 shows a plot of $_s^s$pH against volume % ACN for 13.1 mM MSA compared with the

300 same molar concentrations of TFA and formic acid. Whereas the $_s^s$pH of TFA shows

301 an upturn above about 60 % ACN, that of 13.1 mM MSA remains more or less

302 constant up to 90% ACN. Unfortunately, delta values are not available for

303 concentrations of ACN >90% The ionic strength of these solutions can be estimated

304 from the hydrogen ion concentration given by the $_s^s$ pH and is shown in Fig. 3b.

305 These calculations are approximate, as the graph indicates the ionic strength is

306 somewhat greater than 13.1 mM at some ACN concentrations, which is not possible.

307 Errors can be attributed to the difficulty of accurate measurement of pH in solutions

308 of high ACN content, especially at the low values for this acid. Nevertheless, MSA is

309 clearly a stronger acid than TFA that maintains a higher ionic strength in

310 concentrations of ACN useful for HILIC. We repeated the measurements with a more

311 dilute MSA solution in 95 % ACN ($_w^s$ pH ~ -0.1), which has a more similar acidity to

312 0.1% TFA in the same solvent ($_w^s$ pH ~ +0.5) and is thus less likely to damage the

313 column in long term use. The lower acid concentration should also lessen any

314 suppression effects when using mass spectrometric detection. Hydrolysis is a

315 common problem with bonded reversed phases [24]. Although we did not experience

316 any apparent problem using TFA or MSA, we did not carry out a thorough study of

317 column stability. Nevertheless, as shown by Li and Carr, metal ions (particularly from

318 the frits) can accelerate column degradation, and so the use of columns with

319 polymeric frits may be useful [24]. Furthermore, it is possible that degradation is

320 lessened by the high concentration of organic solvents used in HILIC compared with

321 RP. Fig. 3b indicates that the ionic strength of 3.3 mM MSA remains approximately

322 constant with increasing ACN concentration and exceeds that of 13.1 mM TFA at

323 90% ACN and above.

324   Using this lower concentration of MSA in 95% ACN, Fig. 4 shows the

325 separation of the test mixture on the silica and amide columns. The difference in

326 selectivity from that in TFA (Fig. 2), and the preferential retention of cationic

327   compounds even at the lower pH of MSA is remarkable considering that the
328   ionisation of silanols should be suppressed at this low pH on an inert Type B silica
329   used as the base material for these columns. The correlation coefficient for k TFA vs
330   k MSA using all 15 test compounds was -0.108 and -0.101 for the silica and amide
331   columns respectively, indicating almost no correlation in either case. Preferential
332   retention of cations is demonstrated by the greater retention on the silica column of
333   the somewhat hydrophobic base nortriptyline (log D = +0.9, k = 1.9) compared with
334   the more hydrophilic neutral uridine (log D $_{pH2}$ =-2.1, k = 0.61). Furthermore, the
335   retention of cytosine (k = 3.3, log D = -2.7) and procainamide (k = 25.8, log D = -2.7)
336   was considerably greater than uridine, despite rather similar log D values.
337   While preferential retention of cations is also shown on both columns in AF pH
338   3, the correlation coefficient for k AF pH 3 vs k MSA using all 15 compounds was
339   0.728 and 0.375 for the silica and amide columns respectively, thus indicating
340   important selectivity differences when using MSA. This result is perhaps
341   unsurprising, due to the considerable pH difference in the mobile phases ($_w^s$ pH = -
342   0.1 and 5.9 for MSA and AF pH 3.0 respectively in 95% ACN), and its effect on
343   solute ionisation. For example, pyridine had k ten times greater on both columns
344   using MSA compared with AF pH 3.0. This result could be attributed to its increase in
345   hydrophilicity and capacity for cation exchange on protonation in MSA, compared
346   with its neutral state in AF. Column efficiencies were 15,000-20,000 plates per
347   columnon the amide, and 20000-25000 plates per column on the silica column in 3.3
348   mM MSA with tailing factors below 1.15, indicating good performance. Indeed the
349   tailing factors of 2-NSA and p-XSA were 1.15 on the amide column, which
350   represents a reduction in the values of 1.4 obtained for these solutes when using
351   TFA. This result might be attributed to the greater ionic strength of the MSA mobile
352   phase.
353   The differences in selectivity between TFA and MSA are difficult to explain.
354   The influence of any positive charges which accumulate on the stationary phase in
355   TFA may be emphasised by the rather low ionic strength of TFA solutions in 95%
356   ACN, which nevertheless is sufficient to give good peak shapes for most solutes. In
357   contrast, formic acid solutions have almost no ionic strength in high ACN
358   concentrations (see below). Reduction of the TFA concentration to 0.025% v/v in
359   95% ACN did not however, produce major differences in the retention of the test
360   compounds on the silica column (detailed results not shown). It is possible that

361 adsorption of positively charged artefacts formed by the acid hydrolysis of ACN by

362 TFA may contribute to retention of acidic solutes [26]. However, it is difficult to see

363 why a similar acid hydrolysis should not occur with MSA.

364

365

366 *3.4 Addition of salts or ion pair reagents to the mobile phase.*

367 It is possible that the lower pH of MSA compared with TFA mobile phases is

368 responsible for the selectivity differences shown. Alternatively, it is possible that

369 selective adsorption of methanesulfonate anion could be responsible for cationic

370 retention behaviour of solutes. Thus we added 3.3 mM of its sodium salt (NaMSA) to

371 95% ACN containing 0.1 % TFA. Addition of this salt to the TFA mobile phase

372 produced no change in its pH ($_w^s$ pH = 0.5); Fig. 5 shows the separation of 8 of the

373 test compounds on both columns. Comparison with the chromatograms with MSA

374 alone in 95 % ACN (Fig. 4) shows a similar selectivity although the retention of the

375 base procainamide is considerably reduced. Indeed the k (MSA) vs k (NaMSA) for all

376 15 compounds on the silica and amide columns were well correlated (R= 0.952 and

377 0.8696 respectively). This result suggested that indeed selective adsorption of

378 methylsulfonate ion could be causing the selectivity differences.

379 To investigate this possibility further, we studied the effect of addition of the

380 more hydrophobic salt sodium hexanesulfonate (NaHSA) on the separation of the

381 silica column. If the salt anion was incorporated into the immobilised water layer,

382 perhaps providing cation exchange sites, then selective retention of basic

383 compounds could be explained. This incorporation might be expected to be less

384 pronounced for the more hydrophobic HSA anion compared with the MSA anion. Fig.

385 6a shows a plot of k for the 15 test compounds on the silica column using 3.3 mM

386 NaMSA in 95% ACN/0.1% TFA compared with the same mobile phase using 3.3 mM

387 NaHSA in 95% ACN/0.1% TFA. While the basic compounds (blue markers) are

388 indeed less retained in NaHSA, it appears that retention is quite highly correlated

389 (R= 0.938) in these two mobile phases, suggesting there are no fundamental

390 differences in the selectivity.

391 Furthermore, if adsorption of MSA anion was responsible for retention of

392 cationic solutes, it might be possible to change the selectivity by use of an ion pair

393 agent of different charge. Fig. 6b shows a similar k vs k plot for the silica column

394 comparing retention with NaMSA with trimethylammonium chloride (TMAC), with

395   either salt added to 95% ACN /0.1 % TFA. If adsorption of this reagent on the

396   column surface / incorporation into the water layer occurs, then retention of acidic

397   compounds should be increased in TMAC. However, retention in these two reagents

398   was both very similar and highly correlated (R= 0.994). These results suggest a

399   rather non-specific effect of addition of these salts on the unusual selectivity

400   exhibited in TFA mobile phases, rather than specific adsorption or inclusion of the

401   reagent in the water layer. It is possible that the increase in ionic strength of the

402   solution counteracts the effects of positive charges formed in the presence of TFA.

403   The same interpretation of the increased ionic strength of (3.3 mM) of pure

404   methanesulfonic acid solutions in 95 % ACN compared with (13.1 mM) TFA (see Fig.

405   3b) might explain the lack of anionic retention effects in the former, through

406   increased screening of column charges. Finally, the importance of the effect of ionic

407   strength of the mobile phase is demonstrated in Fig. 7 which shows the separation of

408   the test mixture on the BEH silica column using 0.1% formic acid in 95% ACN. The

409   hybrid structure of this column material results in a low concentration of acidic silanol

410   groups, and thus might conceivably give better peak shapes in formic acid than other

411   types of silica column previously investigated [11]. However, while the peak shapes

412   of the neutral compounds (3 and 4) and the weak acid (7, uncharged in this mobile

413   phase) are good, the peaks of the stronger acids and bases (1,2,5,6,8) are

414   considerably distorted. Formic acid solutions have extremely low ionic strength in

415   mobile phases of high ACN content (see Fig. 3b). Addition of 3.3 mM NaMSA to the

416   formic acid mobile phase (see Fig. 7b) considerably increases the ionic strength and

417   resulted in excellent peak shapes (N = 17,000-25,000 with USP tailing factor < 1.15)

418   for all compounds.

419

420   **4.   Conclusions**

421   Cation retention effects are superimposed on the normal partition and adsorption effects

422   found for silica-based HILIC columns operated with typical salt mobile phases (e.g.

423   ammonium formate), even when the aqueous component of the mobile phase has a

424   low pH. The pH, when measured in the aqueous/organic mobile phase is

425   considerably higher, encouraging ionisation of silanol groups on the underlying silica.

426   This is despite the concomitant effect of the organic solvent in rendering silanol

427   groups somewhat less acidic. When TFA is substituted as mobile phase additive,

428   modern Type B phases show predominately anion exchange properties instead,

429 resulting in enhanced retention of strongly acidic probes and low retention or even
430 exclusion of some bases. As TFA is soluble even in pure ACN, very high retention of
431 acidic probes can be achieved through combined hydrophilic/anionic retention
432 processes. In TFA, the significant cation retention properties of silica hydride phases
433 (Type C) are moderated, but not removed as for the Type B phases. A plastic amide
434 column (PEEK with PEEK frits) and a system washed with the metal complexing
435 agent EDTA also showed anionic solute retention in TFA, indicating that metal ions
436 are unlikely to be the source of these retention sites. It seems possible that
437 incorporation of hydronium ions in the immobilised water layer, or even further
438 protonation of silanols to give positive sites, could be responsible for this behaviour.

439 Substitution of MSA for TFA on amide and silica columns gave markedly
440 different selectivity compared with TFA with preferential cation exchange properties.
441 In part due to the wide difference in pH of the mobile phase between MSA and AF
442 buffered mobile phases, considerable differences in selectivity result between these
443 two systems. As MSA is compatible with mass spectrometry detection, these
444 selectivity differences may be useful in manipulating HILIC separations. Peak
445 shapes in MSA were excellent and for some compounds were better than those
446 obtained in TFA.

447 Methane sulfonate salts added to an ACN/ TFA mobile phase produced rather
448 similar selectivity to use of MSA in aqueous ACN alone. The absence of a pH
449 change on this addition precludes differences in pH between MSA and TFA being
450 responsible for the selectivity differences produced by these two acids. Use of a less
451 hydrophilic salt (hexanesulfonate) did not result in marked selectivity differences; nor
452 did the addition of the oppositely charged ion pair reagent TMAC. It was therefore
453 proposed that it is the increased ionic strength of these salt solutions which
454 neutralises the effect of any surface charges that may be produced by TFA. The
455 increased ionic strength of MSA solutions compared with TFA may also explain the
456 absence of anionic retention effects in the former acid.

457 The importance of maintaining the ionic strength in HILIC separations was
458 shown by the poor peak shape obtained with ionogenic compounds in FA containing
459 mobile phases. Dramatic improvements in peak shape were obtained by addition of
460 methanesulfonate salt to this mobile phase, which considerably increases its ionic
461 strength.

462

5.   References

[1] I. Kohler, R.J.E. Dirks, M. Giera, The rise of hydrophilic interaction chromatography in untargeted clinical metabolomics., LC.GC Europe 29 (2016) 60-75.

[2] A. Periat, I.S. Krull, D. Guillarme, Applications of hydrophilic interaction chromatography to amino acids, peptides, and proteins, J. Sep. Sci. 38 (2015) 357-367.

[3] Y. Guo, Recent progress in the fundamental understanding of hydrophilic interaction chromatography (HILIC), Analyst 140 (2015) 6452-6466.

[4] M.R. Euerby, J. Hulse, P. Petersson, A. Vazhentsev, K. Kassam, Retention modelling in hydrophilic interaction chromatography, Anal. Bioanal. Chem. 407 (2015) 9135-9152.

[5] D.S. Bell, R.E. Majors, Retention and selectivity of stationary phases in HILIC, LC.GC (N. America) 33 (2015) 90-101.

[6] D.V. McCalley, Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography, J. Chromatogr. A 1217 (2010) 3408-3417.

[7] S.M. Melnikov, A. Holtzel, A. Seidel-Morgenstern, U. Tallarek, A Molecular Dynamics Study on the Partitioning Mechanism in Hydrophilic Interaction Chromatography, Angew. Chem. Int. Edit. 51 (2012) 6251-6254.

[8] L. Novakova, L. Havlikova, H. Vlckova, Hydrophilic interaction chromatography of polar and ionizable compounds by UHPLC, Trac-Trend. Anal. Chem. 63 (2014) 55-64.

[9] H. Vlckova, K. Jezkova, K. Stetkova, H. Tomsikova, P. Solich, L. Novakova, Study of the retention behavior of small polar molecules on different types of stationary phases used in hydrophilic interaction liquid chromatography, J. Sep. Sci. 37 (2014) 1297-1307.

[10] Y. Guo, R. Shah, Detailed insights into the retention mechanism of caffeine in hydrophilic interaction chromatography, J. Chromatogr. A 1463 (2016) 121-127.

[11] D.V. McCalley, Study of retention and peak shape in hydrophilic interaction chromatography over a wide pH range, J. Chromatogr. A 1411 (2015) 41-49.

[12] M. Kosmulski, Positive Electrokinetic Charge of Silica in the Presence of Chlorides, J. Colloid Interface Sci. 208 (1998) 543-545.

501 [13] M. Kosmulski, The pH dependent surface charging and points of zero charge.

502 VI. Update, J. Colloid Interface Sci. 426 (2014) 209-212.

503 [14] K.G. Janssen, T.H. Hoang, J. Floris, J. de Vries, N.R. Tas, J.C. Eijkel, T.

504 Hankemeier, Solution titration by wall deprotonation during capillary filling of silicon

505 oxide nanochannels, Anal. Chem. 80 (2008) 8095-8101.

506 [15] J.J. Pesek, M.T. Matyska, R.I. Boysen, Y.Z. Yang, M.T.W. Hearn, Aqueous

507 normal-phase chromatography using silica-hydride-based stationary phases, Trac-

508 Trend. Anal. Chem. 42 (2013) 64-73.

509 [16] J. Soukup, P. Jandera, Adsorption of water from aqueous acetonitrile on silica-

510 based stationary phases in aqueous normal phase liquid chromatography, J.

511 Chromatogr. A 1374 (2014) 102-111.

512 [17] C. Kulsing, Y. Nolvachai, P.J. Marriott, R.I. Boysen, M.T. Matyska, J.J. Pesek,

513 M.T.W. Hearn, Insights into the Origin of the Separation Selectivity with Silica

514 Hydride Adsorbents, J. Phys. Chem. B 119 (2015) 3063-3069.

515 [18] C.E. Wujcik, J. Tweed, E.P. Kadar, Application of hydrophilic interaction

516 chromatography retention coefficients for predicting peptide elution with TFA and

517 methanesulfonic acid ion-pairing reagents, J. Sep. Sci. 33 (2010) 826-833.

518 [19] A. Kumar, J.C. Heaton, D.V. McCalley, Practical investigation of the factors that

519 affect the selectivity in hydrophilic interaction chromatography, J. Chromatogr. A

520 1276 (2013) 33-46.

521 [20] D.V. McCalley, Is hydrophilic interaction chromatography with silica columns a

522 viable alternative to reversed-phase liquid chromatography for the analysis of

523 ionisable compounds?, J. Chromatogr. A 1171 (2007) 46-55.

524 [21] J.C. Heaton, J.J. Russell, T. Underwood, R. Boughtflower, D.V. McCalley,

525 Comparison of peak shape in hydrophilic interaction chromatography using acidic

526 salt buffers and simple acid solutions, J. Chromatogr. A 1347 (2014) 39-48.

527 [22] J.C. Heaton, D.V. McCalley, Some factors that can lead to poor peak shape in

528 hydrophilic interaction chromatography, and possibilities for their remediation, J.

529 Chromatogr. A 1427 (2016) 37-44.

530 [23] J.J. Pesek, M.T. Matyska, S.M. Fischer, Improvement of peak shape in

531 aqueous normal phase analysis of anionic metabolites, J. Sep. Sci. 34 (2011) 3509-

532 3516.

533   [24] L.Ma, P.W. Carr, Loss of bonded phase in reversed-phase liquid

534   chromatography and practical ways to improve column stability, Anal. Chem. 79

535   (2007) 4681-4686.

536   [25] L.G. Gagliardi, C.B. Castells, C. Rafols, M. Roses, E. Bosch, delta Conversion

537   parameter between pH scales ((s)(w)pH and (s)(s)pH) in acetonitrile/water mixtures

538   at various compositions and temperatures, Anal. Chem. 79 (2007) 3180-3187.

539   [26] X.-R. Kei, C. Gong, Y.L. Zhang, X. Xu, Chromatographia 79 (2016) 1257-1262.

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6.   Legend to Figures

Fig. 1 Separation of 1=p-XSA; 2= 2-NSA; 3= thiourea; 4 =uracil; 5 = nortriptyline; 6 = procainamide; 7=4-OH benzoic acid; 8 =cytosine. Blue = basic solutes; green = neutral solutes; red = acidic solutes. Column: BEH HILIC 3.5 μm particles, 15 x 0.46 cm, temperature 30 ^{o}C, injection volume 5 μL, detection UV at 215 nm, flow rate 1 mL/min. Mobile phase 95% ACN containing 5 mM ammonium formate pH 3.0

Fig. 2 Separation of test compounds on a) BEH silica and b) BEH amide (both 3.5 μm particles, 15 x 0.46 cm) c) Silica hydride 4 μm particles, 25 x 0.46 cm Mobile phase 0.1 % TFA in 95 % ACN. Other conditions and peak identities as Fig. 1.

Fig.3 (a) Plot of true thermodynamic $_s^s$pH and (b) Plot of ionic strength versus acetonitrile concentration (v/v) for different acid solutions.

Fig. 4   Separation of test compounds on a) BEH silica and b) BEH amide. Mobile phase 3.3 mM methanesulfonic acid in 95 % ACN. Other conditions and peak identities as Fig. 1.

Fig. 5 Separation of test compounds on a) BEH silica and b) BEH amide. Mobile phase 3.3 mM NaMSA in 95 % ACN containing 0.1% TFA. Other conditions and peak identities as Fig. 1.

Fig. 6 k vs k plots for 15 test compounds on BEH silica column. Blue diamonds = basic, Red triangles = acidic, Green circles = neutral solutes. a) k 3.3 mM NaHSA in 95% ACN 0.1% TFA vs k 3.3 mM NaMSA in 95%ACN 0.1 % TFA. b) k 3.3 mM TMAC in 95% ACN 0.1% TFA vs k 3.3 mM NaMSA in 95% ACN 0.1 % TFA.

Fig. 7 Separation of test compounds on BEH silica. a) Mobile phase 0.1 % formic acid in 95% ACN. b) Mobile phase 0.1 % formic acid in 95% ACN with 3.3 mM NaMSA. Other conditions and peak identities as Fig. 1.
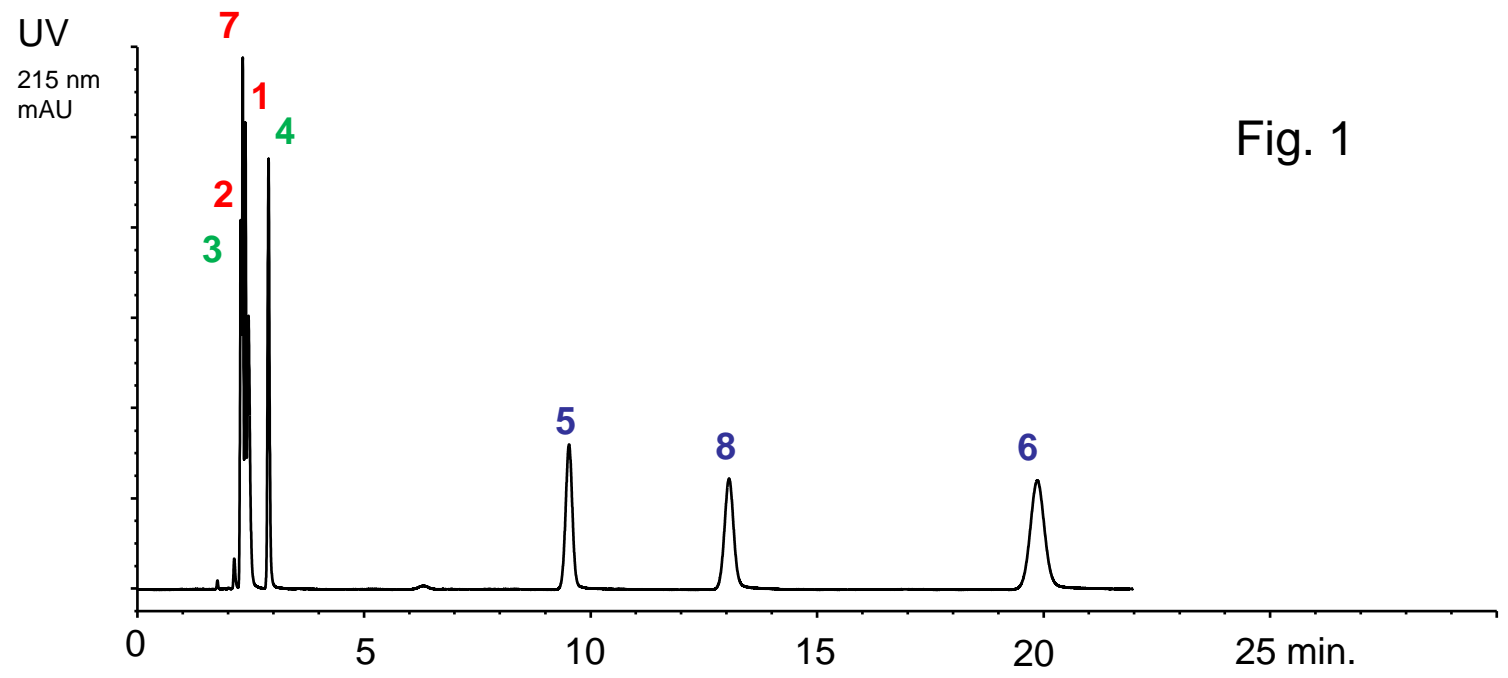
Figure 1

Fig. 1
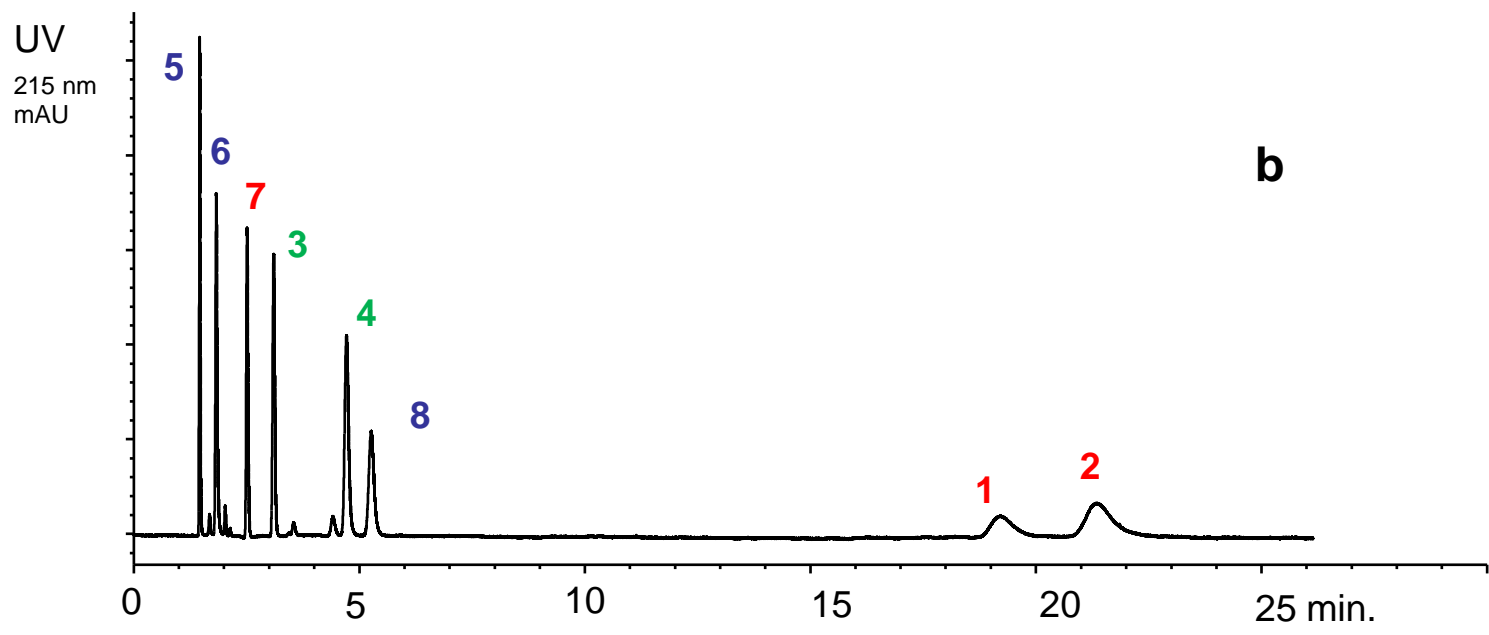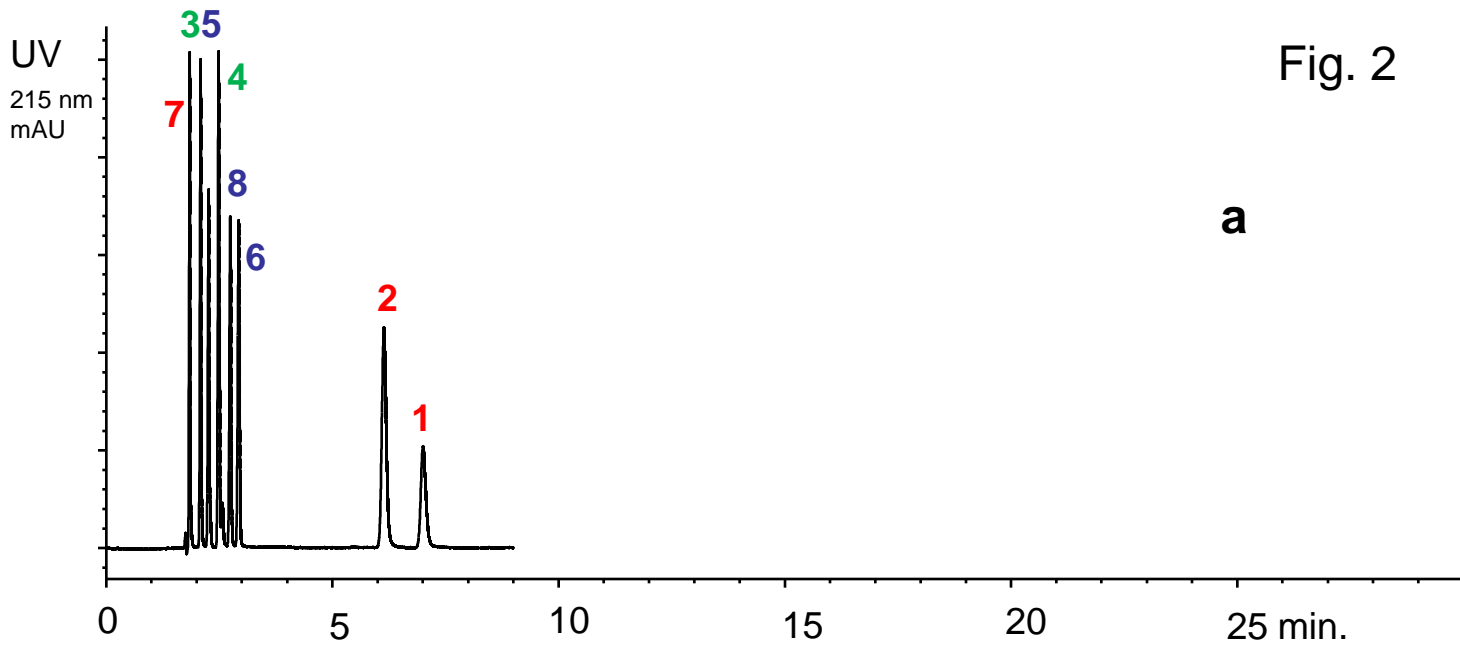
Fig. 2

Fig 2c
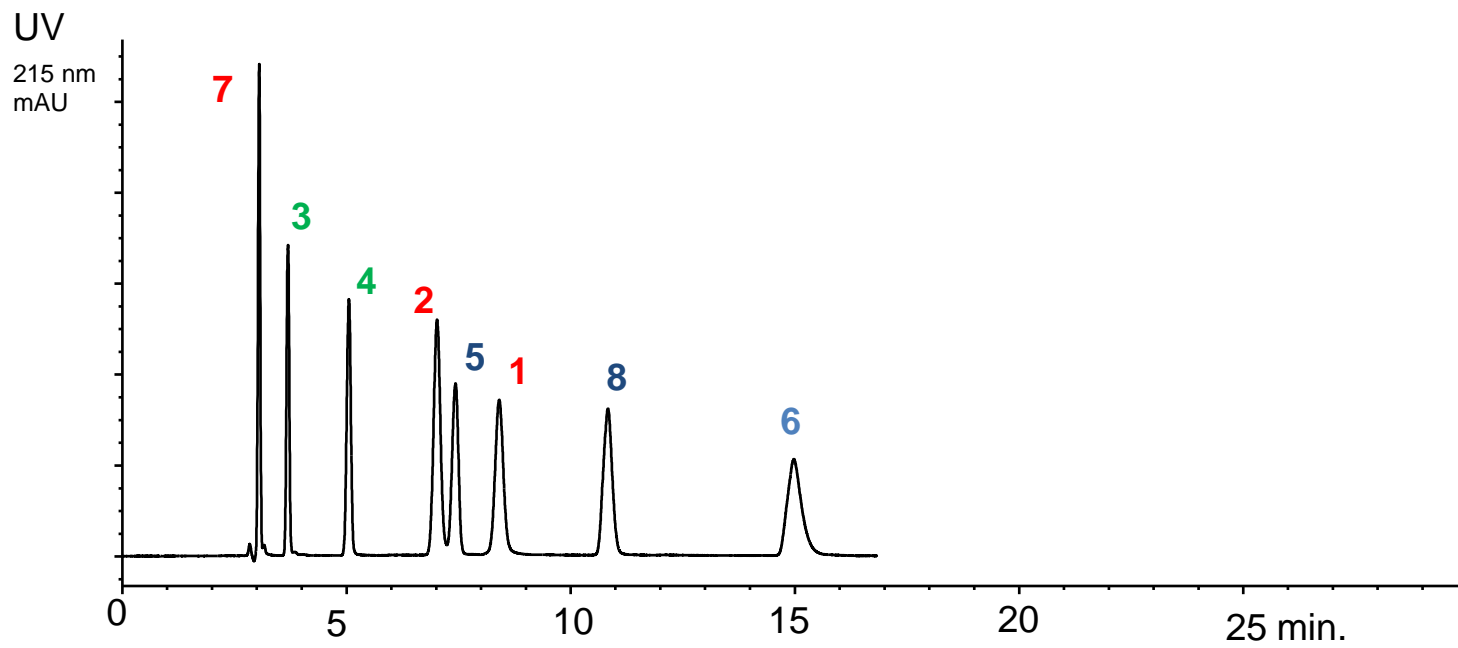
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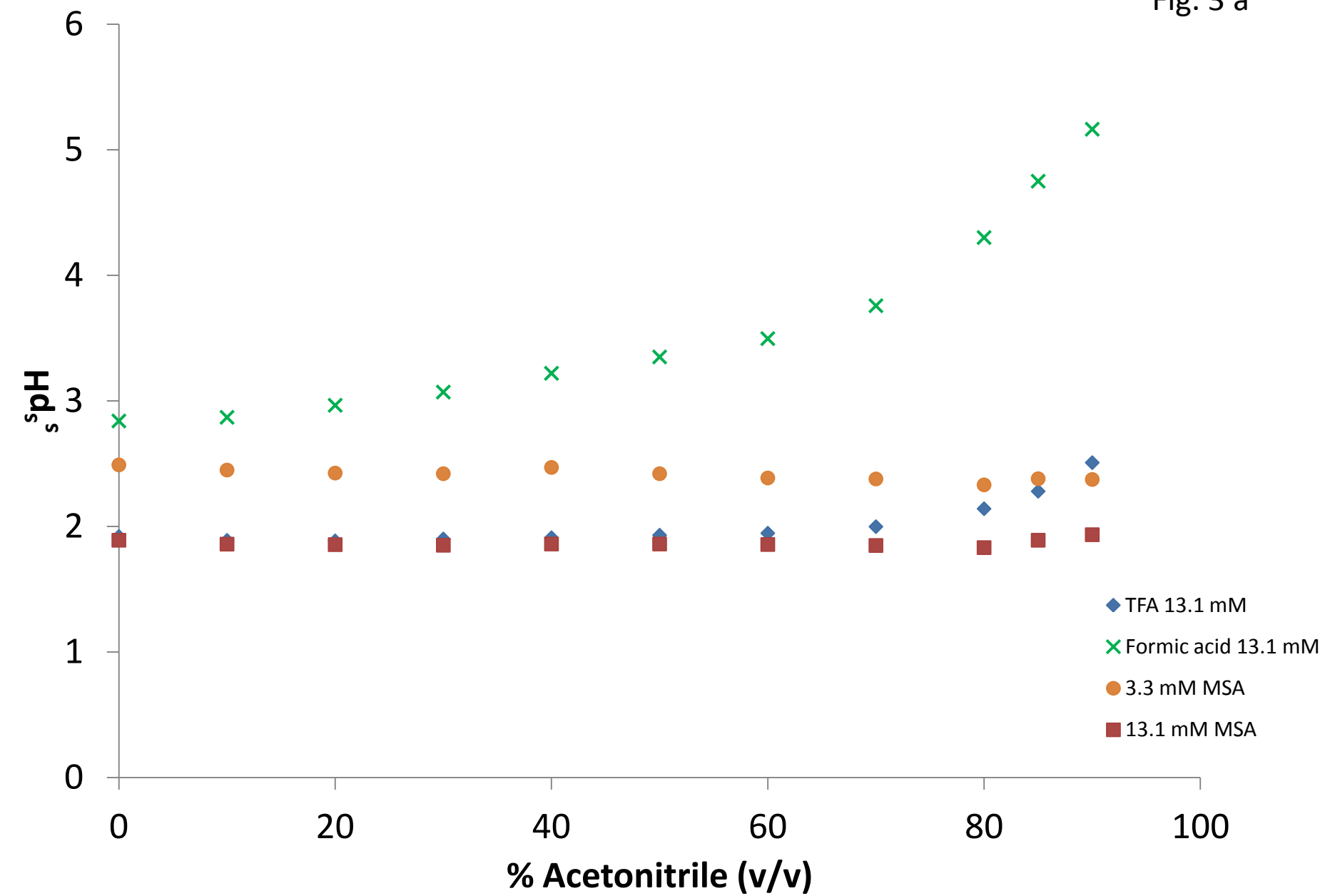
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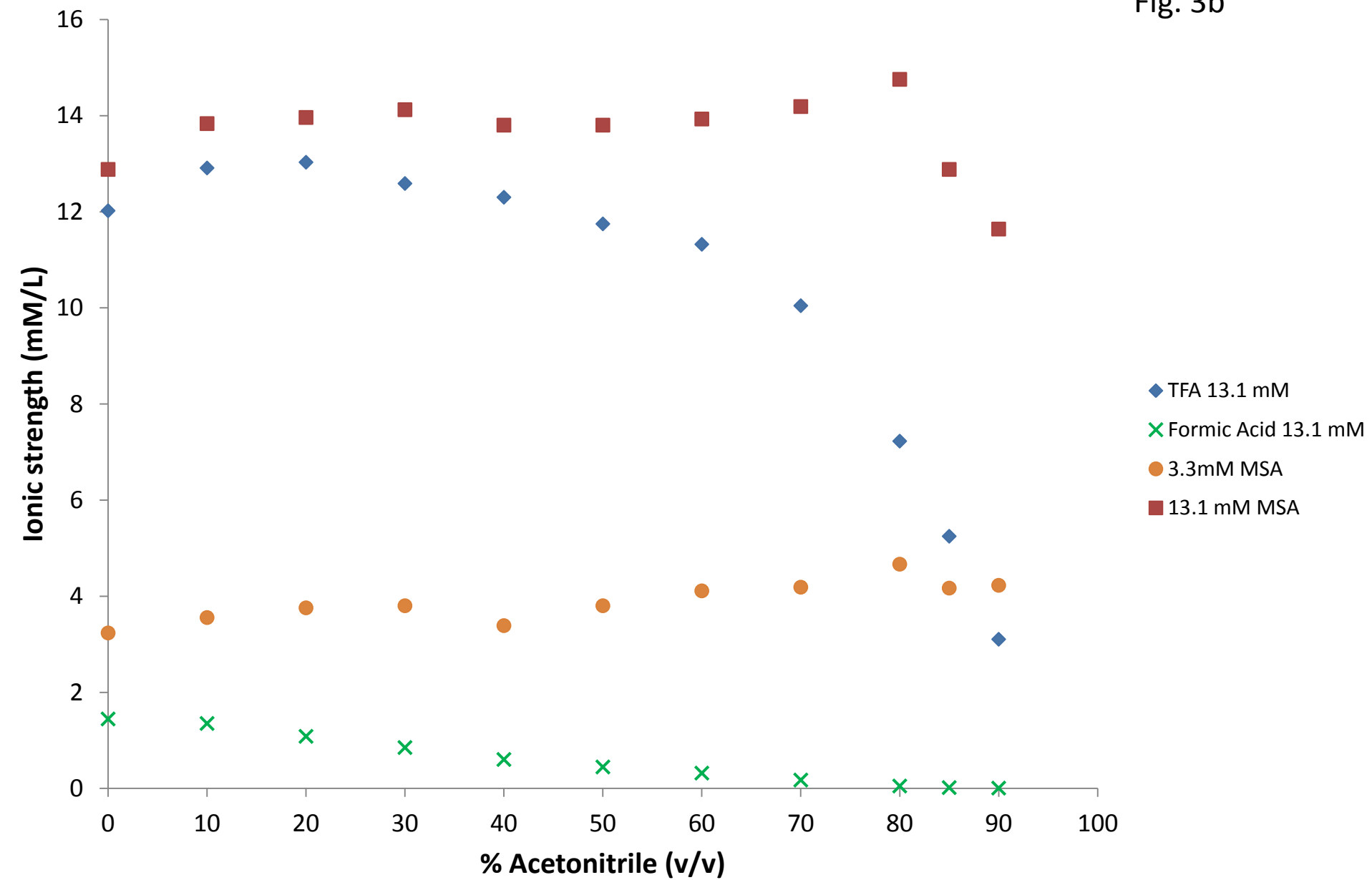
Figure 4

Fig. 4
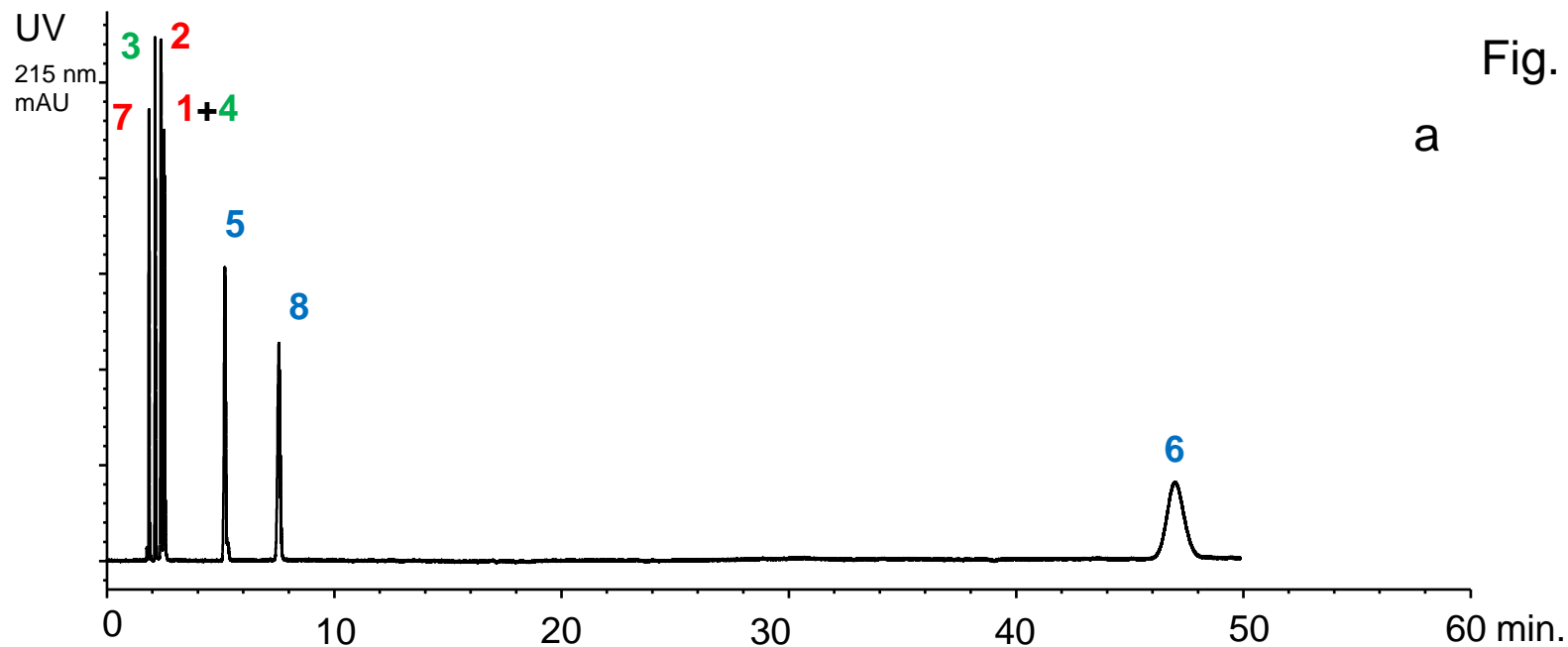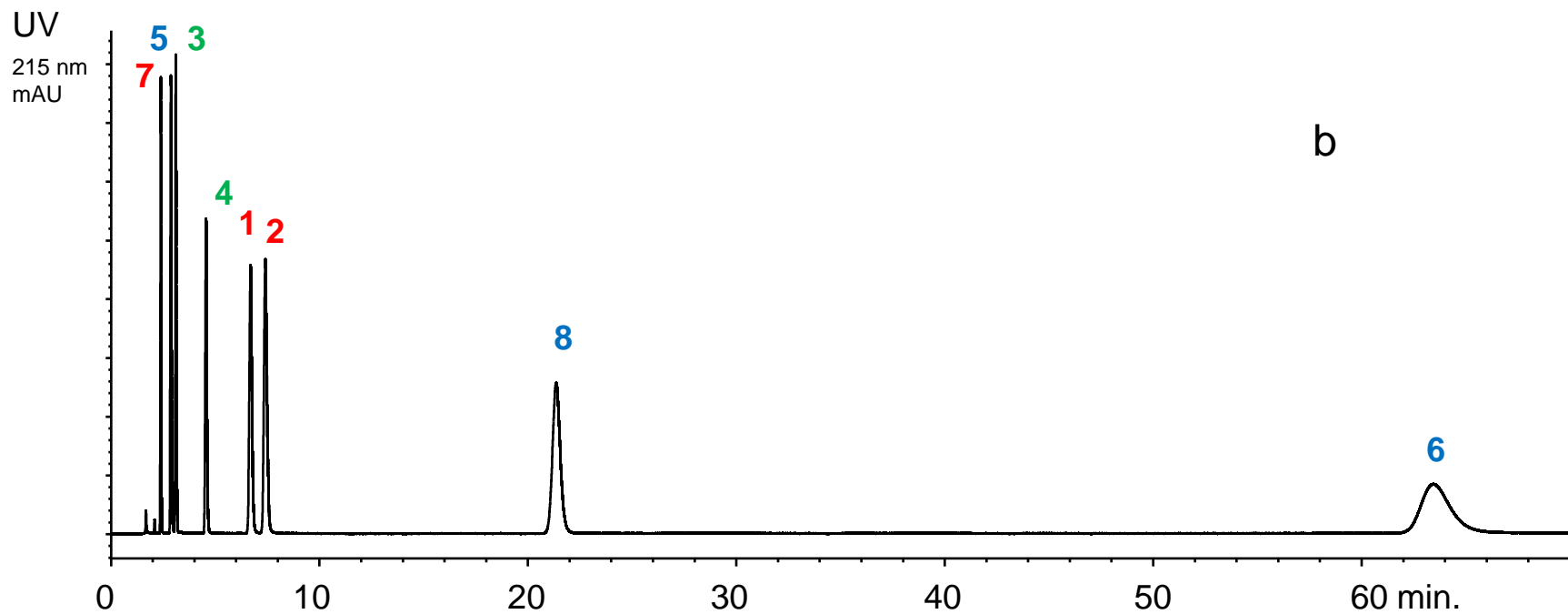
Figure 5

Fig.5
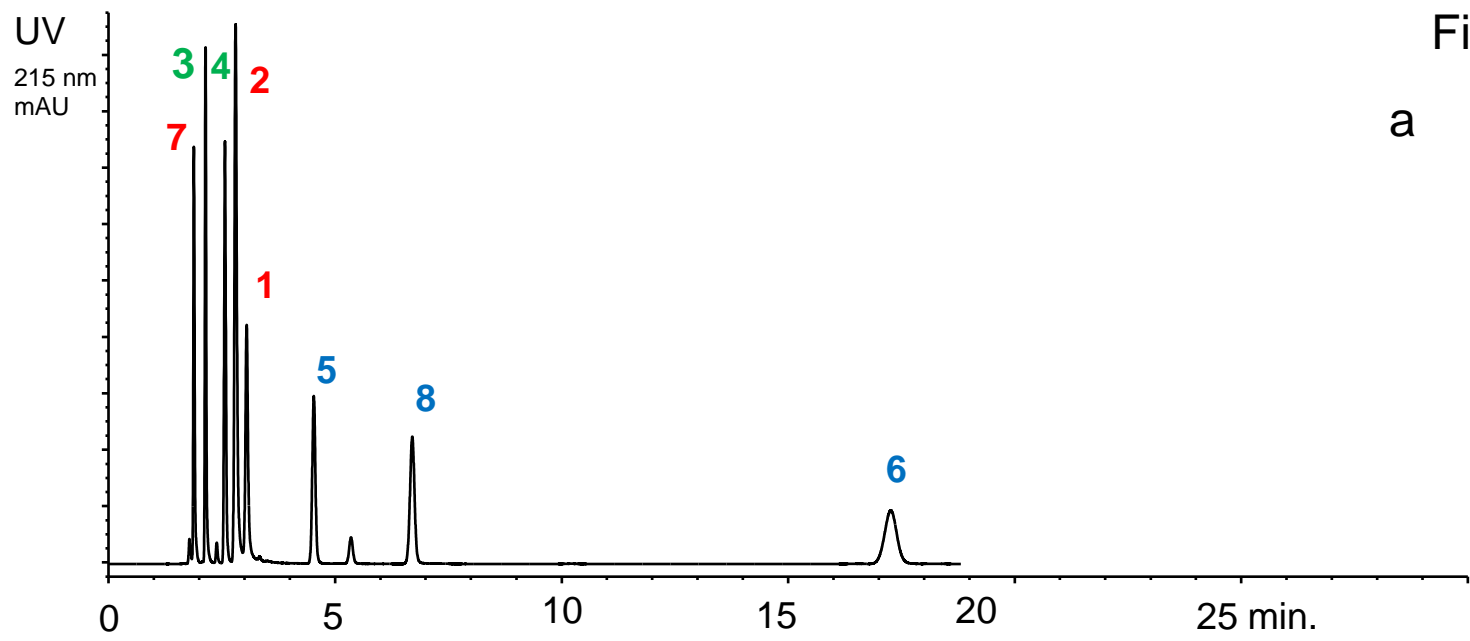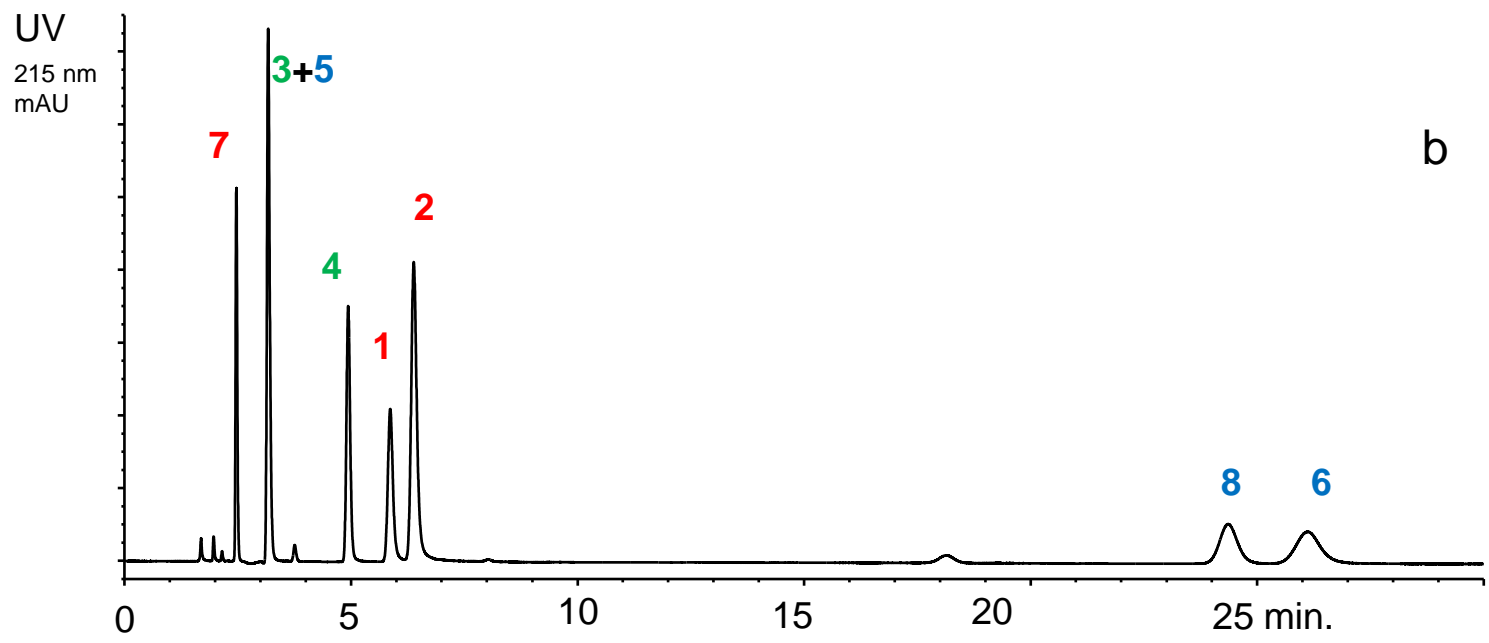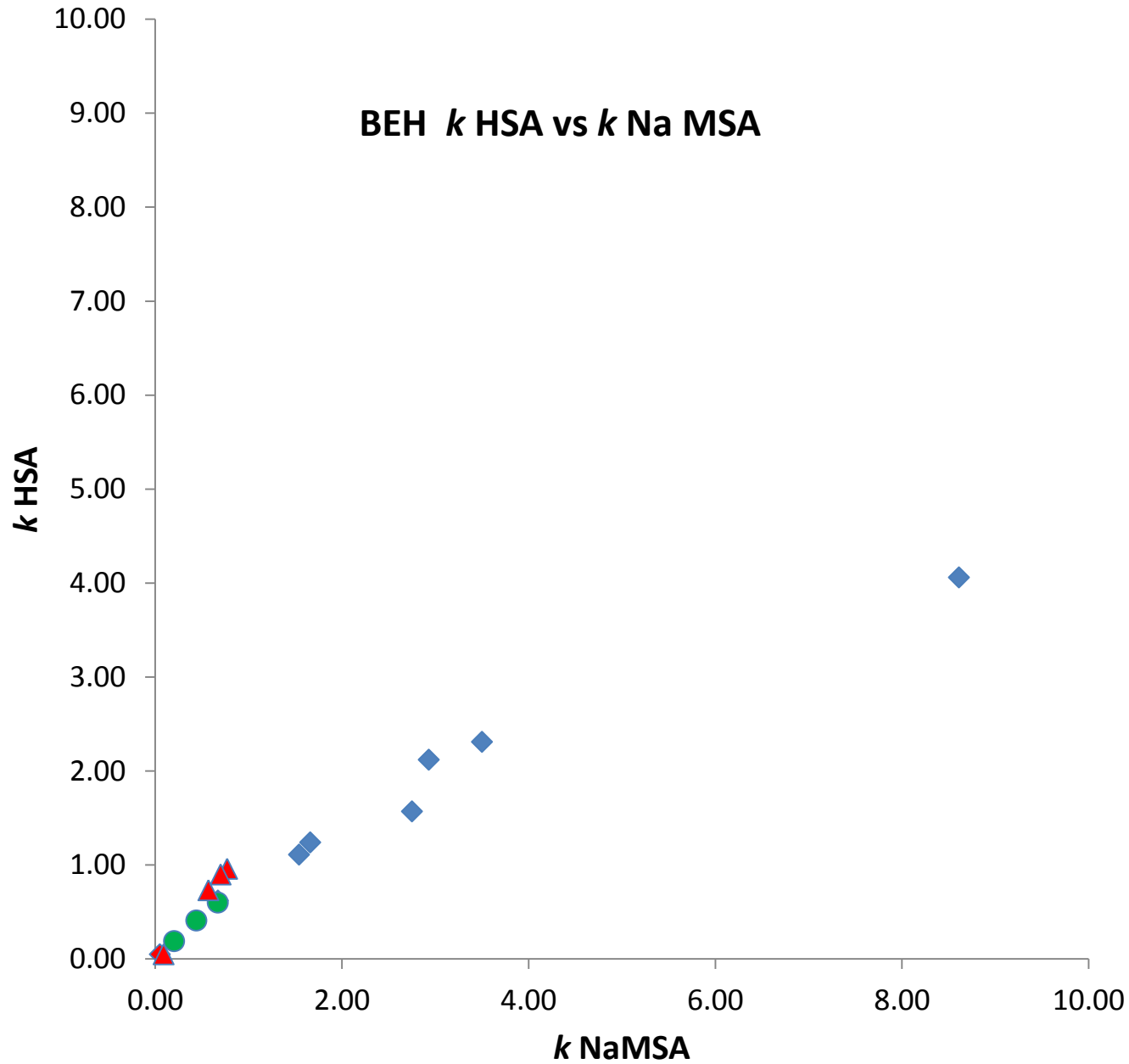
Fig. 6a

Fig 6b

Figure 7

Fig. 7

Table 1
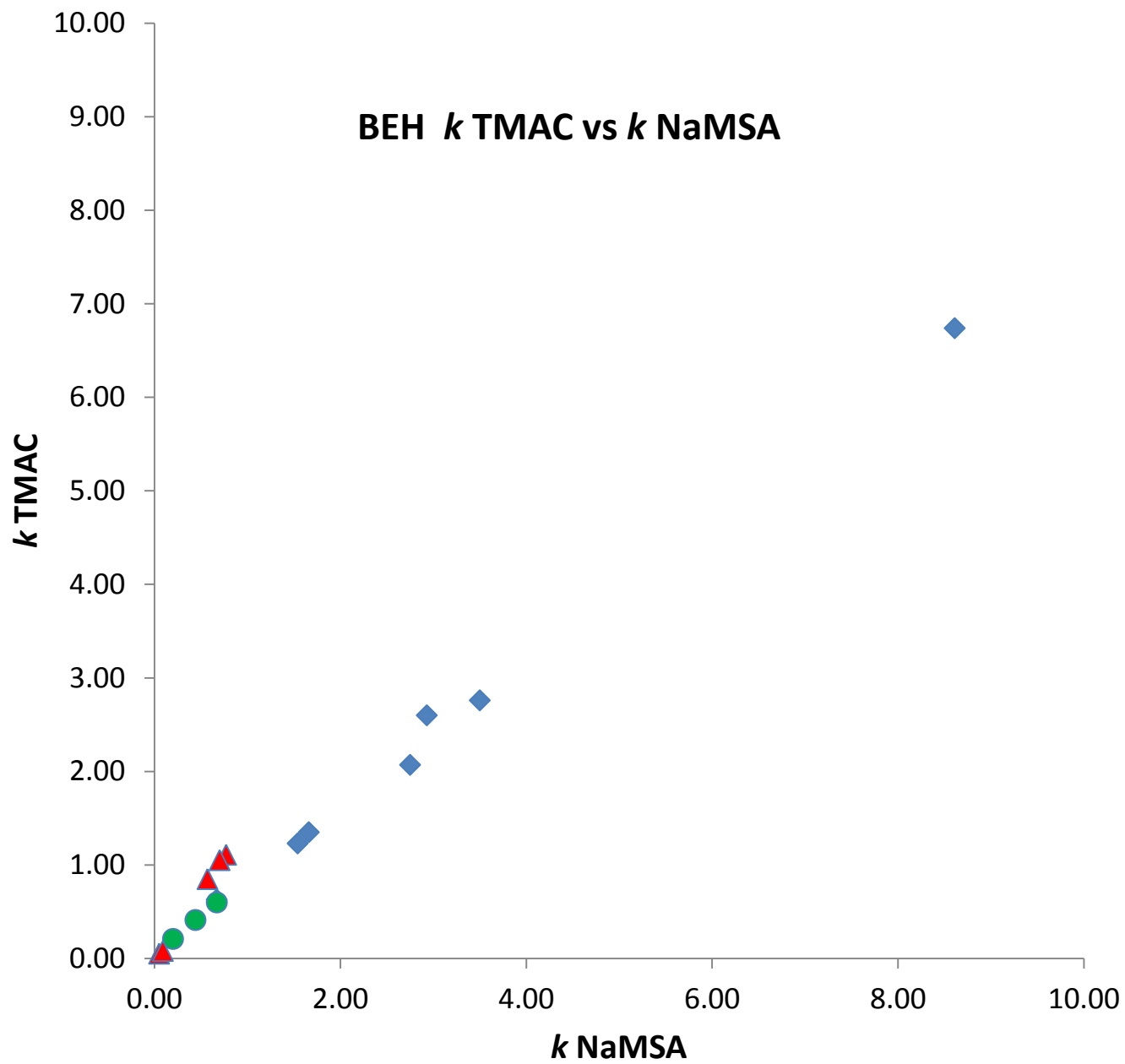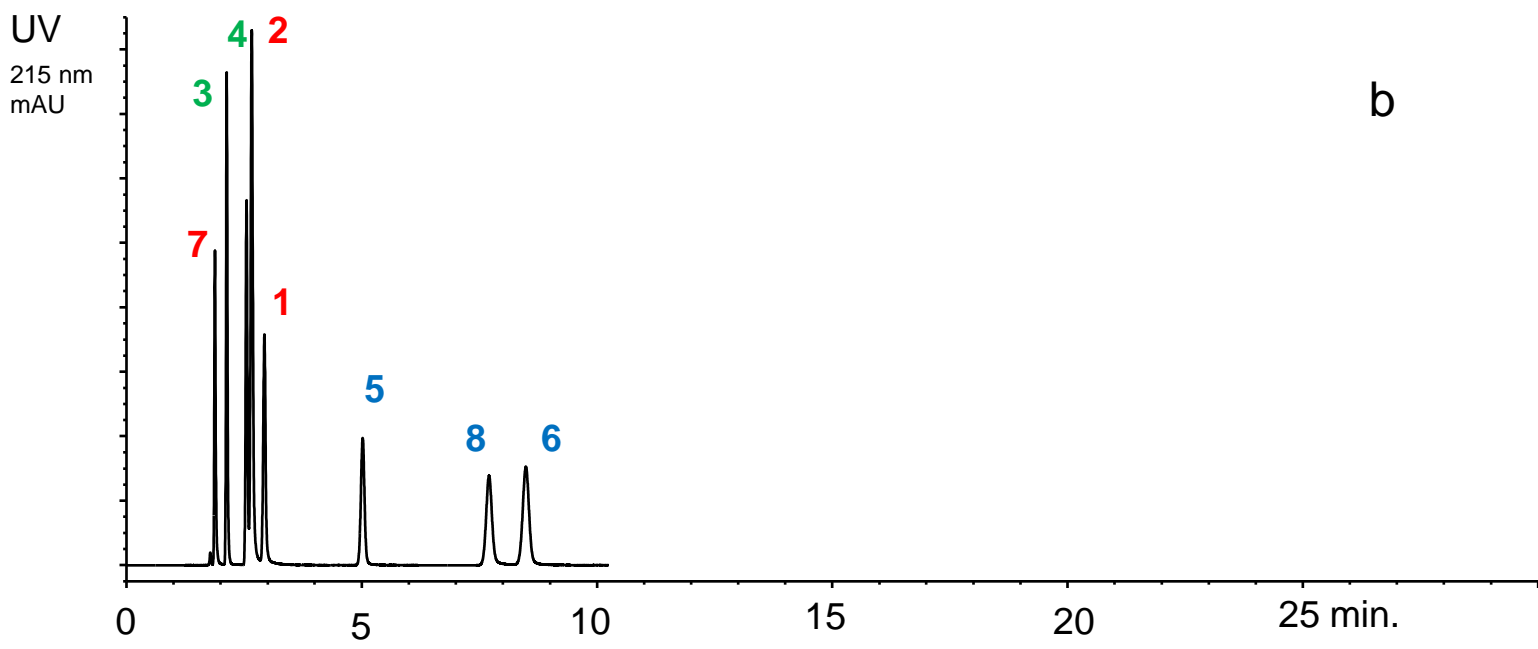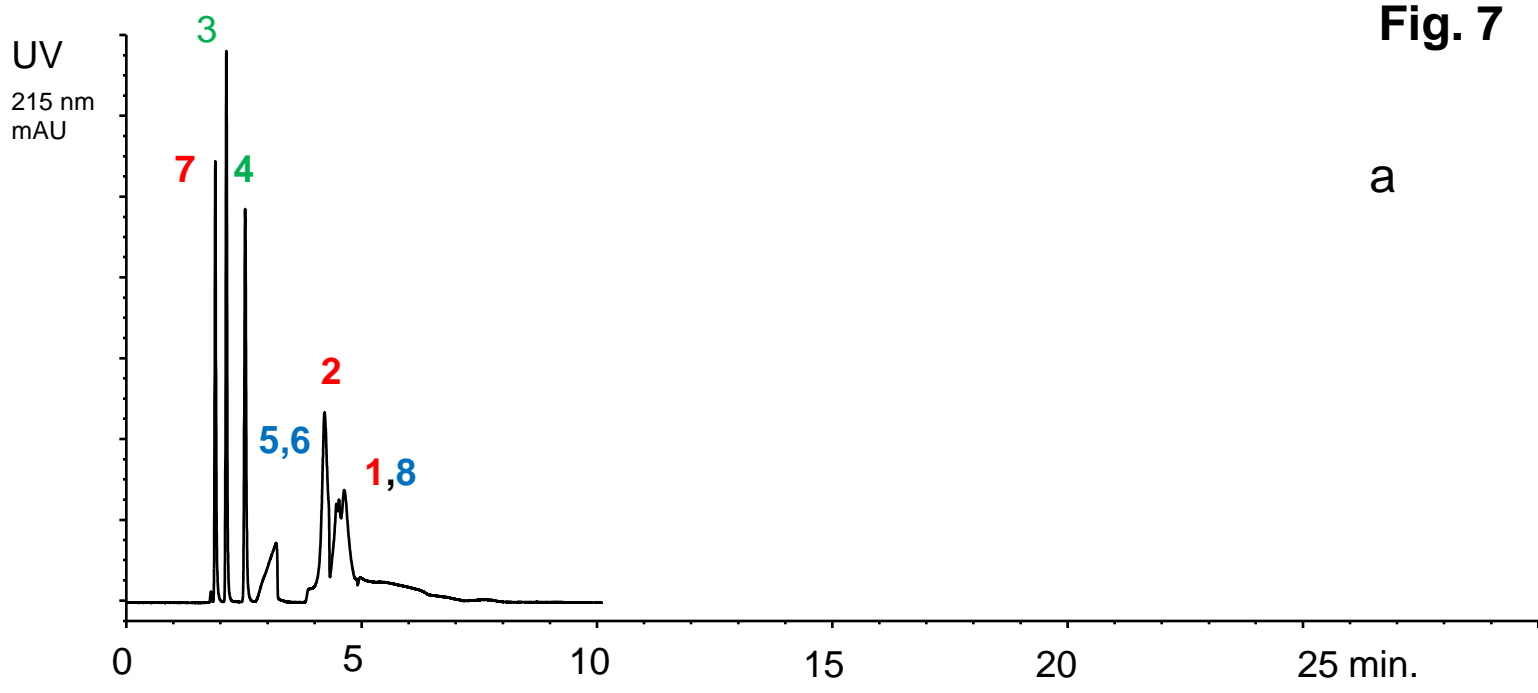
Table 1 *k* values of neutral, acidic and basic probes on BEH silica column in acetonitrile-water mixtures of varying concentration each containing 0.1 % TFA.

Solute	*k* 99% ACN (v/v)	*k* 98% ACN	*k* 97% ACN	*k* 95%ACN
uracil	0.66	0.56	0.50	0.41
nortriptyline	-0.28	-0.12	+0.02	0.29
procainamide	-0.21	+0.04	+0.24	+0.66
2-NSA	>50	>50	13.9	2.5
p-XSA	>50	>50	16.4	3.0

Supplimentary Table